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Defect of Adenovirus Type 12 Replication in Hamster Cells: Absence of Transcription of Viral Virus-Associated and L1 RNAs

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The nonpermissive interaction of hamster cells with human adenovirus type 12 (Ad12) is characterized by a total block of Ad12 DNA replication and late transcription, whereas most of the early functions of Ad12 DNA can be transcribed. Ad2 can replicate in hamster cells. The replication and late transcription defects of Ad12 DNA can be complemented to a certain extent by the E1B functions of Ad2 DNA. This complementation fails, however, to lead to the synthesis of the late Ad12 proteins and to the assembly of infectious virions. It will now be demonstrated that the Ad12 L1 (late genes of group 1) and virus-associated (VA) RNAs are not transcribed in hamster cells. Synthesis of these RNAs in productively infected human cells or Ad2-infected hamster cells is readily detectable by S1 nuclease protection experiments and Northern (RNA) blotting. Similarly, the Ad2-transformed hamster cell line BHK-Ad2E1 fails to complement L1 and VA RNA syntheses after superinfection with Ad12. However, Ad12 infection of the Ad5-transformed hamster cell line BHK297-C131 leads to the transcription of the Ad12 L1 and VA segments. This difference in complementation by the two transformed hamster cell lines might be accounted for by functions in the segment of Ad5 DNA extending between map units 30 and 40 and persisting in the Ad5-transformed hamster cells or by hamster host cell functions which might be operative in cell line BHK297-C131 but not in BHK-Ad2E1 or BHK-21 hamster cells.

The nonproductive interaction of human adenovirus type 12 (Ad12) with Syrian hamster cells has been studied in detail. Ad12 virions cannot replicate in hamster cells (6, 7, 26). There is a total block of Ad12 DNA replication in this host system (7, 9, 11), although virions are taken up into the cytoplasm and viral DNA enters the nucleus and can be integrated into the host genome (6, 8). In hamster cells, most of the early viral functions are transcribed into mRNA (16–18) which can be expressed in a cell-free transcription system (10). In contrast, transcription of the late Ad12 genes in hamster cells cannot be detected (13, 14, 17). Thus, Syrian hamster cells fail to provide a permissive milieu for late gene expression and replication of Ad12 DNA. There is evidence that this defect is due, at least in part, to the failure of the major late promoter of Ad12 DNA to function in hamster cells, whereas the same promoter is operative in human cells and the major late promoter of Ad2 DNA is capable of functioning in both hamster and human cells (31). Similarly, Ad2 is capable of replicating in both cell types (7, 21).

In Syrian hamster (BHK-21) cells which are doubly infected with Ad2 and Ad12 or with Ad5 and Ad12, Ad12 DNA can replicate, but Ad12 proteins or virions are not synthesized. The complementing functions in the Ad2 or Ad5 genome (which help to overcome in part the replication defect of Ad12 DNA in hamster cells) reside, at least in part, in the E1B segment of Ad2 or Ad5 DNA (14). Similar conclusions have been documented in experiments with Ad12-superinfected Ad2- or Ad5-transformed hamster cells, which contain (in an integrated form) and constitutively express the viral E1 region (13, 14). In this latter system, it has also been shown that the transcription of late Ad12 genes is complemented by Ad2 or Ad5 functions (13, 14). These findings suggest that a second defect rendering the Ad12 genome inactive in replication and late transcription in hamster cells can be attributed to the viral E1B region.

In an attempt to understand in more detail the nature of the deficiencies that incapacitate the Ad12 genome in Syrian hamster cells, we have continued to investigate this virus-host system. In the present report, it is demonstrated that the virus-associated (VA) RNA (19) and L1 genes of Ad12 DNA are not transcribed in hamster cells. This documentation is important, because it has previously been shown that the virally coded VA RNA is essential in mediating the translation of the late viral messenger RNAs (23, 27). The transcription of VA RNA is at least partly controlled by the early region E1A of adenovirus DNA (3, 12). Most of the mRNAs of the Ad12 E1 region are expressed in hamster cells. Hence, it is conceivable that Ad12 VA RNA was made in hamster cells. It has also been reported elsewhere that some of the L1-encoded proteins in Ad2 DNA can be expressed early in the productive infection cycle (1, 5, 25, 28). It is therefore possible that L1 functions of Ad12 DNA may be expressed in nonpermissive hamster cells, although L1 is dominated by the major late promoter of adenoviruses. Moreover, there is evidence that L1 functions are involved in the assembly of infectious virions in the productive infection cycle (T. B. Hasson, P. D. Soloway, W. Doerfler, and T. Shenk, submitted for publication).

All techniques used in this study were described previously (13–15, 30). BHK-21 hamster or HeLa human cells were grown in Dulbecco modified Eagle medium containing 10% fetal bovine serum. Ad2 or Ad12 was propagated on human HeLa cells growing in monolayer or suspension cultures. Methods for virus purification and viral DNA extraction were reported earlier (7). For experiments with BHK-21 or HeLa cells, which grew in monolayers, multiplicities of infection of about 400 PFU of adenovirus per cell were employed. The characteristics of cell lines BHK297-C131 (13, 29) and BHK-Ad2E1 (30) were described elsewhere. Briefly, cell line BHK297-C131 carried the left terminal 18.7% and a submolar fragment between map

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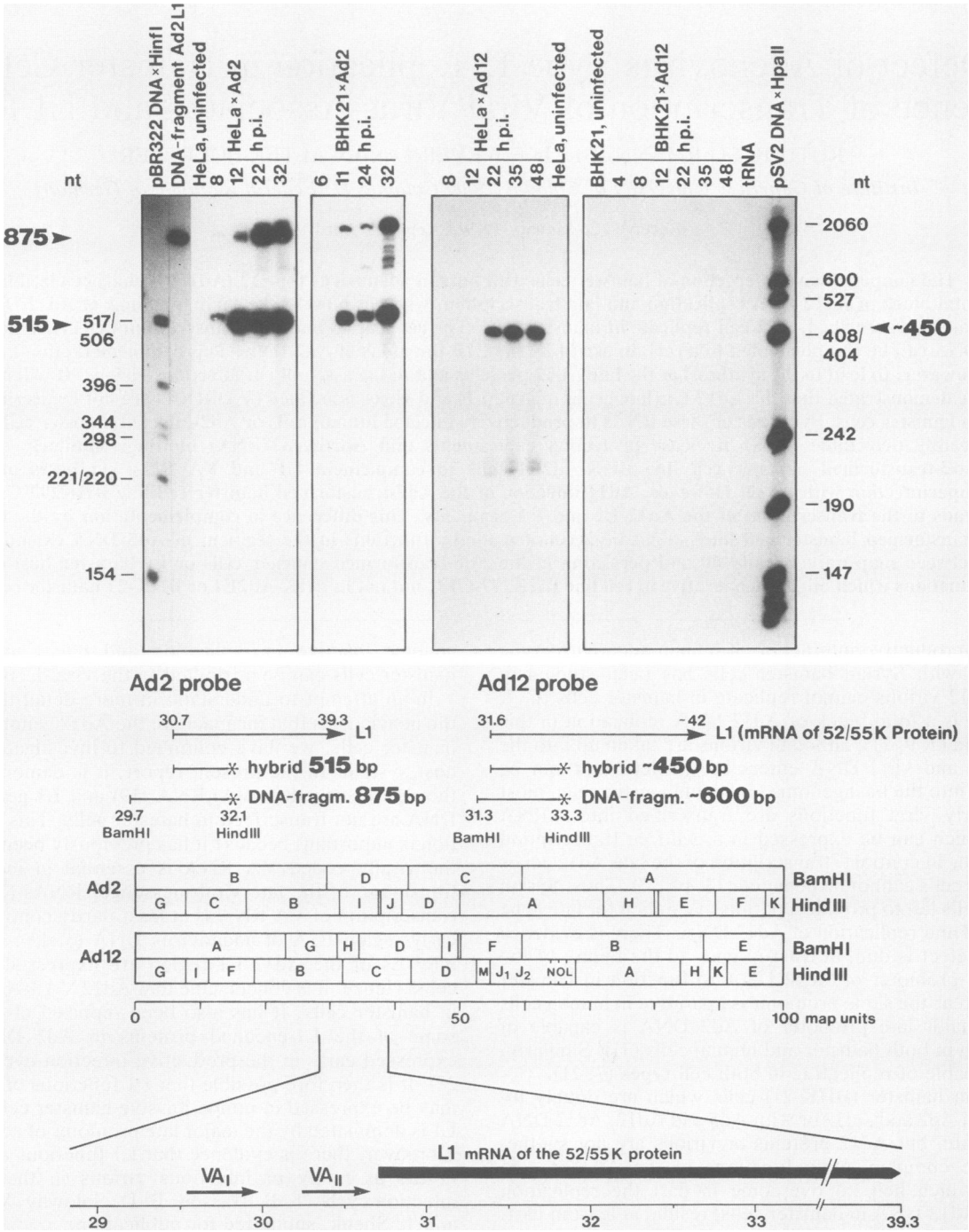

FIG. 1. S1 nuclease protection analyses of Ad2- or Ad12-specific L1 RNAs form HeLa and BHK-21 cells. The probe for Ad2 RNAs was prepared as follows. The *Hin*dIII B fragment of Ad2 DNA, which had been cloned in pBR322 DNA, was excised with *Hin*dIII, 5′ terminally labeled with polynucleotide kinase (20) and [γ-^{32}P]ATP, and cleaved with *Bam*HI. The 875-base-pair (bp) *Bam*HI-*Hin*dIII fragment (map units 29.7 to 32.1) was purified by polyacrylamide gel electrophoresis and used for the hybridization experiment. The probe for Ad12 RNAs was prepared as follows. The pBR322 DNA-cloned *Bam*HI H fragment of Ad12 DNA was *Hin*dIII cleaved at map unit position 33.3, 5′ terminally labeled as described above, and then cut with *Bam*HI at map unit position 31.3. The generated ^{32}P-labeled fragment with a length of about 600 base pairs was gel purified and used as a hybridization probe. The marker lane contained the 5′-terminal ^{32}P-labeled *Hin*fI fragments of pBR322 DNA or the *Hpa*II fragments of pSV2 DNA. Fragment lengths in the autoradiographs are indicated in nucleotides (nt). The *Bam*HI and *Hin*dIII restriction maps of Ad2 and Ad12 DNAs are also presented for orientation. The lengths of the ^{32}P-labeled DNA fragments (*) are indicated. The enlargement details the locations of the VA1, VA2, and L1 regions between map units 29.5 and 39.3 on the Ad2 genome.

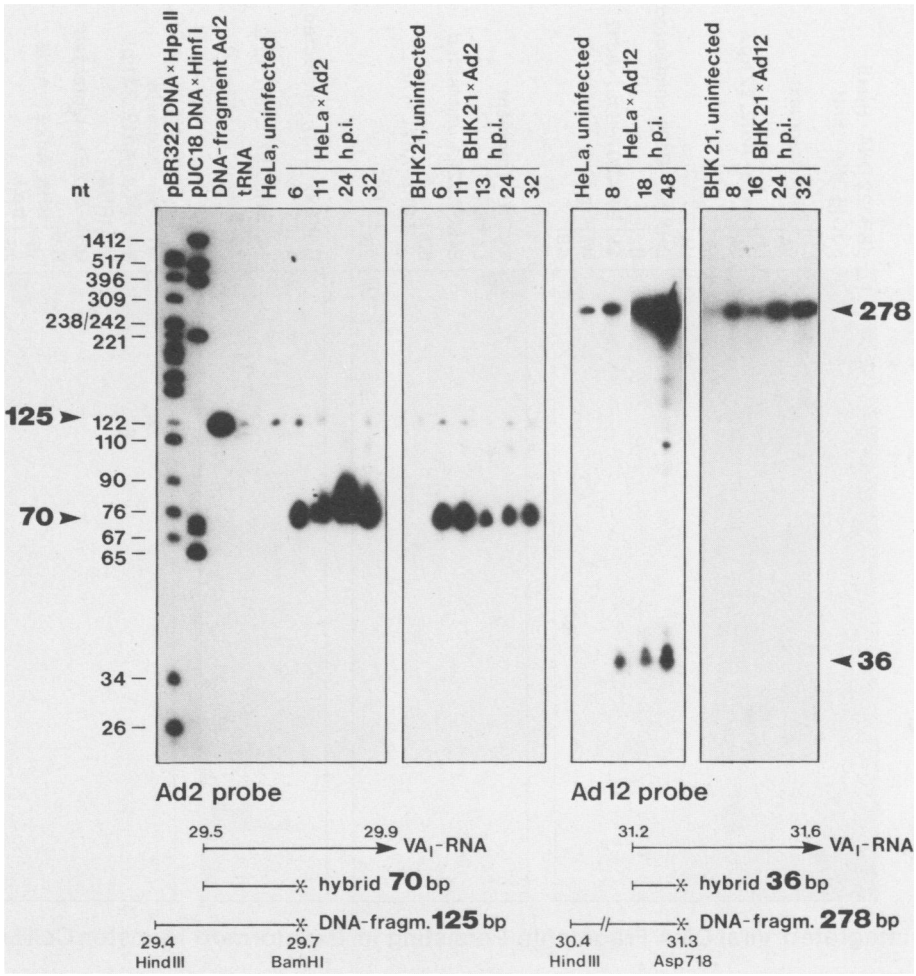

FIG. 2. S1 nuclease protection analyses of Ad2- or Ad12-specific VA RNAs form HeLa and BHK-21 cells. The experimental design and conditions are described in the text. Since the synthesis of VA RNA instead of L1 RNA was to be probed, hybridization fragments different form those used in the experiments illustrated in Fig. 1 were resolved, because of their smaller sizes, by electrophoresis on 7% polyacrylamide gels containing 7% urea. The probe for Ad2 RNAs was prepared as follows. The pUC18 DNA-cloned *Xba*I-*Bal*I fragment of Ad2 DNA was *Bam*HI cleaved at map unit position 29.7, 5′ terminally ^{32}P-labeled as described in the legend to Fig. 1, and cut at the *Hin*dIII site of the pUC18 vector, which was next to map unit position 29.4 of Ad2 DNA. The 125-base-pair (bp) *Hin*dIII-*Bam*HI fragment was isolated by polyacrylamide gel electrophoresis. The probe for Ad12 RNAs was prepared as follows. The pUC18 DNA-cloned *Bam*HI-*Bgl*II fragment of Ad12 DNA was *Asp* 718 cleaved at map unit position 31.3, 5′ terminally ^{32}P-labeled, and cut at the *Hin*dIII site of the pUC18 vector, which was next to map unit position 30.4 of Ad12 DNA. The 278-base-pair *Hin*dIII-Asp 718 fragment was gel purified. Fragment lengths are indicated in nucleotides (nt). The lengths of the ^{32}P-labeled fragments (*) are indicated. The marker fragments were the 5′-terminal-^{32}P-labeled *Hpa*II fragments of pBR322 DNA or the *Hin*fI fragments of pUC18 DNA.

positions 30 and 40 of the Ad5 genome in an integrated form. In cell line BHK-Ad2E1, the left 17.1% of the Ad2 genome was fixed by integration into the hamster cell genome (cf. maps in Fig. 3).

For RNA transfer hybridization (Northern [RNA] blotting) experiments (2, 22) and S1 nuclease analyses of RNAs (4, 15), standard techniques were employed. HeLa or BHK-21 cells were mock infected with phosphate-buffered saline or infected with Ad2 or Ad12 at about 400 PFU per cell, as described above. At various times after the infection of BHK-21 cells with Ad12, the total cytoplasmic RNA was isolated by the hot-phenol method (24) and analyzed for the presence of VA- or L1-specific sequences. RNAs from Ad12-infected human HeLa cells (the permissive system) were investigated as positive control. Similarly, RNAs from the Ad2-transformed hamster cell line BHK21-Ad2E1 or the Ad5-transformed hamster cell line BHK297-C131 were studied at various times after superinfection with Ad12. RNAs from cells permissively infected with Ad2 were also used as positive controls. The RNAs were electrophoretically fractionated on formaldehyde-agarose gels and transferred to nitrocellulose filters by Northern blotting. The exact derivations of these RNAs were also determined by the nuclease S1 mapping procedure. Unselected cytoplasmic RNA (10 μg) was hybridized (15) to DNA fragments (5 to 20 ng, equivalent to 10,000 cpm), as detailed in the legends to Fig. 1 and 2, and treated with nuclease S1 as described elsewhere (30). The protected DNA fragments were resolved by electrophoresis on 4 to 7% polyacrylamide gels containing 7 M urea. After being dried, the gels were autoradiographed on Kodak XAR5 film. The map locations of the VA and L1 genes on the Ad2 genomes are shown in Fig. 1. Equally precise genetic maps for Ad12 DNA are not presently available.

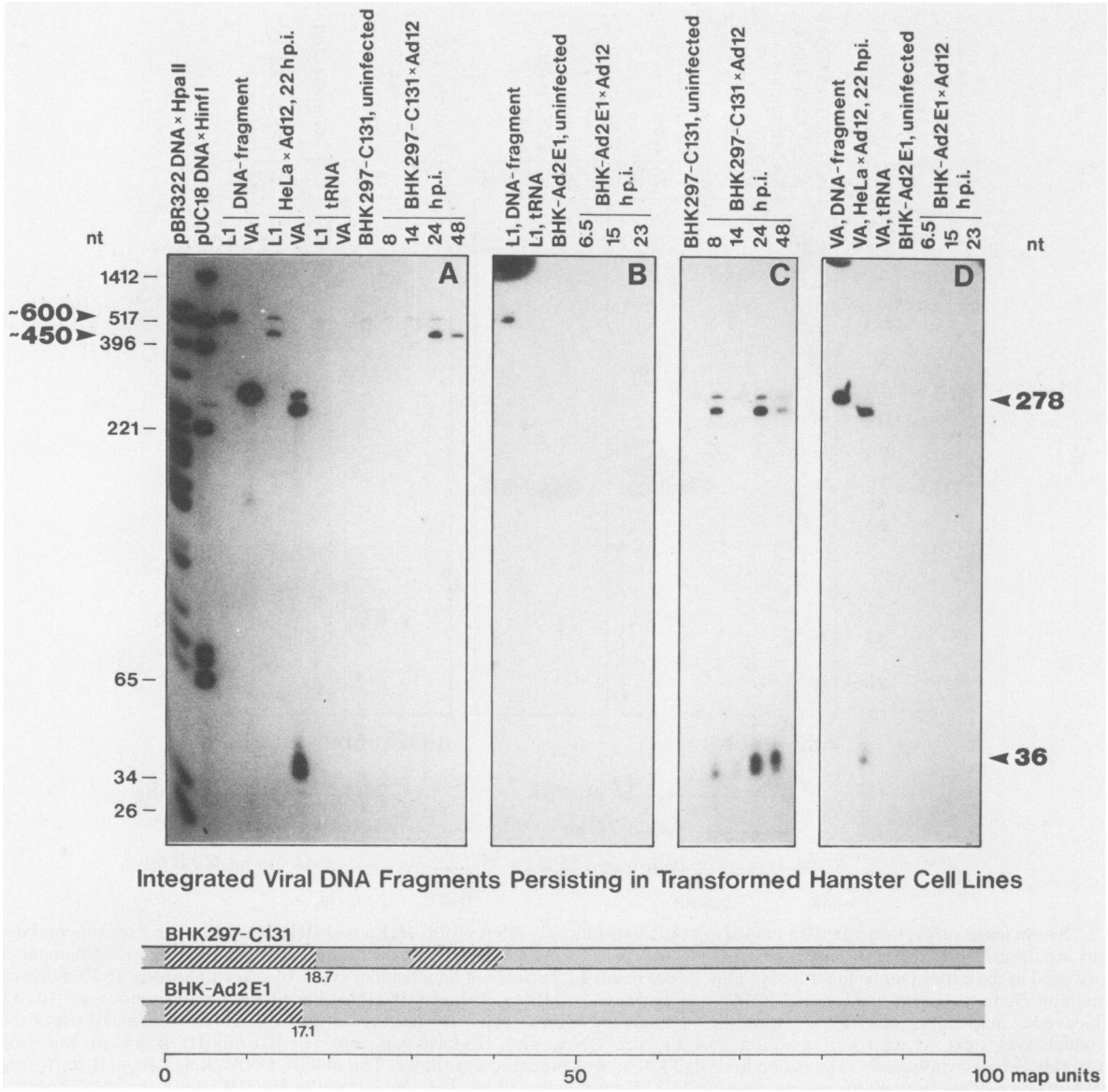

FIG. 3. S1 nuclease protection analyses of Ad2- or Ad12-specific L1 or VA RNA in Ad12-infected cell lines BHK297-C131 or BHK-Ad2E1. The hybridization probes used were as detailed in the legends to Fig. 1 and 2. In the hybridization analyses, either the L1 or the VA probe of Ad2 or Ad12 DNA was used as detailed in the text. Fragment lengths are indicated in nucleotides (nt). The maps below the autoradiograms indicate those sections of the Ad5 or Ad2 genome that persist in an integrated form in the two transformed hamster cell lines (▨). Marker fragments were used as described in the legend to Fig. 2.

In RNA transfer (Northern blotting) experiments using the ^{32}P-labeled *Hin*dIII I fragment of Ad2 DNA as hybridization probe, L1-specific RNAs of Ad2 were found starting 4 h postinfection (p.i.) of HeLa cells and 11 h p.i. of BHK-21 cells (data not shown). In similar experiments after Ad12 infection, hybridization with the ^{32}P-labeled *Pst*I F fragment of Ad12 DNA revealed Ad12 L1-specific RNAs starting 22 h p.i. of HeLa cells. However, in Ad12-infected BHK-21 cells, Ad12 L1-specific RNA signals were not detected.

These findings were corroborated by subjecting the RNAs screened for Ad2- or Ad12-specific signals to the more sensitive S1 nuclease analysis. The results of this study are presented in Fig. 1, which also details the sizes of the Ad2 L1 and Ad12 L1 probes, their respective map locations, and the lengths of the ^{32}P-labeled DNA fragments which were protected by hybridization to RNA. With the RNAs isolated from human HeLa cells after infection with Ad2 or Ad12, L1-specific signals of protected, labeled DNA fragments were detectable, indicating that viral L1 RNAs were synthesized in either productive system. By this method, Ad2-specific L1 RNA was first recognized 8 h p.i., and Ad12-specific L1 RNA was recognized 22 h p.i. Similarly, in BHK-21 cells productively infected with Ad2, evidence for the presence of L1-specific RNA was observed starting 11 h p.i. RNA from uninfected HeLa or BHK-21 cells did not contain adenovirus-specific sequences (Fig. 1, control lanes). Between 4 and 48 h after infection of BHK-21 cells with Ad12, L1-specific RNA was not detectable. These data were reproduced several times. It is concluded that in nonpermissive BHK-21 cells, Ad12 infection fails to elicit

the synthesis of L1-specific RNA. In Ad12-infected human cells or Ad2-infected HeLa or BHK-21 cells, L1-specific RNAs are synthesized.

The RNAs from Ad2- or Ad12-infected HeLa or BHK-21 cells were also analyzed for the presence of VA RNA-specific signals (Fig. 2). The results of Northern blotting and hybridization experiments (not shown) revealed VA RNA in Ad12-infected HeLa cells starting 8 h p.i. but failed, in repeated attempts, to detect any trace of Ad12-specific VA RNAs in Ad12-infected BHK-21 cells. These findings were again refined by applying the S1 nuclease protection procedure. The results of these experiments (Fig. 2) demonstrate the presence of VA-specific sequences in RNA from Ad2- or Ad12-infected HeLa cells which were productively infected by these viruses and with RNA from Ad2-infected BHK-21 cells which were also permissive for Ad2 infection. In contrast, Ad12-specific VA RNA was not synthesized in Ad12-infected BHK-21 cells between 8 and 32 h p.i., and VA RNA-protected signals were absent (Fig. 2). These results were reproduced several times.

It is concluded that the protection experiments performed in this study allowed the detection of VA RNAs in productively infected cells. In the abortively Ad12-infected BHK-21 cells, Ad12-specific VA RNA was not produced, even though most of the E1 functions of Ad12 DNA were transcribed (10, 16). It is conceivable that in addition to the E1A functions (3, 12), host cell functions are required to activate the polymerase III-mediated transcription of the Ad12-specific VA RNA. Apparently, BHK-21 cells fail to provide the cellular milieu conducive to Ad12 VA RNA synthesis.

It has been shown elsewhere that Ad5- or Ad2-transformed hamster cell lines, which carry the integrated left viral termini and express them constitutively, can compensate for some of the replication and late transcription defects of Ad12 DNA in hamster cells (13, 14). Therefore, we investigated whether L1 or VA RNA of Ad12 could be produced in Ad5- or Ad2-transformed hamster cells after superinfection with Ad12. The maps of the persisting viral genome fragments in these cell lines are shown in Fig. 3. Cell lines BHK297-C131 and BHK-Ad2E1 were superinfected with Ad12, and the cytoplasmic RNA was analyzed at various times after Ad12 infection for the presence of Ad12-specific L1 and VA signals. These studies were performed as described in the legends to Fig. 1 and 2, and the same Ad2- or Ad12-specific L1 or VA probes as are detailed there were employed for RNA hybridization experiments.

The data demonstrated that Ad12-specific L1 (Fig. 3A) and VA RNAs (Fig. 3C) were synthesized after the Ad5-transformed cell line BHK297-C131 was superinfected with Ad12 but not after the Ad2-transformed cell line BHK-Ad2E1 was infected with Ad12 (Fig. 3B and D). Of course, the uninfected line BHK297-C131 lacked Ad12-specific RNAs. The RNA from Ad12-infected BHK297-C131 or HeLa cells yielded a 250-nucleotide signal with the VA hybridization probe of Ad12 DNA (Fig. 3, band underneath 278-nucleotide signal). These signals were caused by Ad12-specific RNAs which have not been identified. The lack of VA RNA is, therefore, unlikely to be the cause for the failure of late Ad12 mRNAs to be translated into Ad12-specific proteins (14) after superinfection of BHK297-C131 cells with Ad12.

The complementation by cell line BHK297-C131 of the L1 and VA transcription defects of Ad12 DNA in hamster cells may have been mediated by Ad5 functions encoded in the segment extending between map units 30 and 40 which persisted in submolar amounts in that cell line (13) or by host cellular functions which happened to be activated in this transformed hamster cell line but not in the Ad2-transformed cell line or in BHK-21 cells which were devoid of Ad12 DNA.

The results of the analyses described here show that in the abortive Ad12-hamster cell system, both L1 and VA RNA transcriptions are deficient, although production of these RNAs can be readily detected in productively infected cells and in Ad5-transformed hamster cells. VA RNA transcription, at least in Ad2-infected cells, is thought to be controlled by the early viral E1A functions (3, 12). However, in Ad12-infected BHK-21 hamster cells, the available E1A viral functions by themselves are obviously not sufficient for the transcription of L1 and VA RNAs, and hamster cell functions fail to support the transcription of the L1 and VA regions of the Ad12 genome.

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